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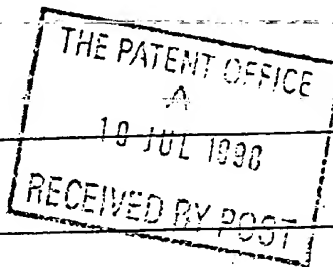
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The University of Nottingham  
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4 Title of the invention

Screening of Neisserial Vaccine Candidates Against  
Pathogenic Neisseria

5 Name of your agent (if you have one)

Swindell & Pearson

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## SCREENING OF NEISSERIAL VACCINE CANDIDATES AGAINST PATHOGENIC NEISSERIA

The present invention relates to vaccine candidates in Pathogenic *Neisseria*, and particularly but not exclusively to a screening system for the identification of CD4<sup>+</sup> T-cell stimulating vaccine candidates in Pathogenic *Neisseria*.

The term "vaccine candidates" is used to refer to peptides which may prove, upon further study, to exhibit some form of vaccine property. In particular, the vaccine candidates discussed below are peptides which stimulate CD4<sup>+</sup> T-cells (T-cells with CD4 marker on them).

The generic name Pathogenic *Neisseria* covers the pathogenic organisms *Neisseria meningitidis* and *Neisseria gonorrhoea*.

*Neisseria meningitidis* (the meningococcus) causes meningitis and overwhelming septicaemia that can kill within hours. It also causes outbreaks of meningococcal disease. *Neisseria gonorrhoea* (the gonococcus) causes gonorrhoea and other invasive diseases, e.g. pelvic inflammatory diseases and septic arthritis.

Although the two neisserial species (*N. meningitidis* and *N. gonorrhoea*) have evolved to colonise and invade different anatomical sites of the human body, they are strongly related and share extensive amount of genetic, immunochemical and other biological properties. They are believed to have evolved from a common ancestor, a view strongly supported by the recently released respective genomic sequence data. The outer membrane structure of the two organisms are very similar with a vast number of outer membrane proteins, including some vaccine candidates, being virtually identical. Recent data suggest that vaccines based on conserved (cross-reactive) immunogenic proteins may protect against both organisms.

The mechanisms responsible for the development of natural immunity to meningococcal disease remain unclear and the currently available capsular polysaccharide (CPS)-based vaccines provide only serogroup-specific and short-lived protection and are not effective in children under two years of age. Additionally, the CPS of serogroup B meningococci, which are responsible for the majority of cases in Europe and America, is only very poorly immunogenic in humans, generating mainly IgM antibodies.

Recovery from meningococcal infection is followed by long lasting immunity and, in the absence of immunodeficiencies, second episodes of meningitis (with homologous or heterologous strains) are extremely rare. This fact indicates that there are non-capsular (cross-reactive) antigens that can stimulate T-cell memory and thus generate a long-lasting and cross-protective immunity.

To achieve an efficient humoral immune response resulting in the production of high affinity IgG antibodies and the generation of memory B lymphocytes (B-cells), help from T lymphocytes (T-cells) is required. However, helper T-cells respond to peptide antigens associated with class II molecules of the major histocompatibility complex (MHC - designated HLA in humans) on the surface of antigen presenting cells. Therefore, they will not be stimulated by purified polysaccharide vaccines (T-cell independent B-cell immunogens). To trigger a strong memory T-cell response when the host confronts the virulent organism, the target B-cell epitope should be expressed along with helper T-cell stimulating epitopes. Identification and characterisation of the peptide epitopes that can best stimulate meningococcal specific CD4<sup>+</sup> T-cells is considered among the priorities that have long been ignored. An ideal meningococcal vaccine must consist of a carefully selected mixture of well-characterised B- and T-cell antigens capable of generating a long lasting immunity.

It appears that meningococcal vaccine candidates will also have the potential to protect against gonococcal disease.

In the following description the term T-cell clones is defined as the population of cells which originate from a single T cell.

In a first aspect, the present invention provides a method of generating T-cell lines and clones specific to neisserial proteins, the method comprising isolating peripheral blood lymphocytes (PBLs) from the peripheral blood of normal donors and patients recovering from neisserial disease, culturing the PBLs with neisserial proteins with or without a proliferation stimulant for a prescribed period, stimulating proliferation of T-cell lines and clones which are specific to neisserial proteins, and maintaining same by regular stimulation.

The neisserial proteins are preferably prepared from *Neisseria meningitidis* and/or *Neisseria gonorrhoea* grown under iron restrictions to induce the expression of iron-regulated proteins.

The peripheral blood is preferably obtained from naturally infected patients at different stages of illness. Preferably the stages include an acute stage (on admission), early convalescence (seven days after admission), late convalescence (six weeks after discharge) and after full recovery (3 months and twelve months after discharge).

Preferably the peripheral blood is heparinised and the PBLs may be isolated therefrom by centrifugation.

Preferably the PBLs are initially cultured in medium containing human serum. Preferably the PBLs are cultured with the neisserial proteins and Interleukin 2 (IL-2) for the prescribed period. Preferably the prescribed period is 3-10 days and may be 5 days.

Preferably IL-2 stimulates the proliferation of the activated T-cell lines and clones. Preferably the T-cell lines and clones are maintained by weekly stimulation. The stimulation may be provided by proteins in the presence of

IL-2 and feeder cells. Preferably the feeder cells are antigen presenting feeder cells and may be autologous Epstein-Barr virus transformed B-lymphocytes (EBVB).

The specificity of the T-cell lines and clones to neisserial proteins is preferably tested prior to storing in liquid nitrogen. Preferably the specificity is tested by measurement of tritiated thymidine incorporation in response to stimulation with neisserial proteins compared to irrelevant antigens. Such an irrelevant antigen may be tetanus toxoid. The phenotype of the T-cell lines and clones are preferably also assessed using flow cytometry and specific monoclonal antibodies. The antibodies are preferably CD4<sup>+</sup>, CD8<sup>+</sup> and  $\alpha/\beta$ - and  $\gamma/\delta$ - T-cell receptor (TCR) specific monoclonal antibodies.

In a second aspect the present invention provides a method of detecting CD4<sup>+</sup> T-cell stimulating proteins, the method comprising fractionating neisserial proteins and testing the ability of said proteins to stimulate proliferation of T-cell lines and clones.

Preferably the T-cell lines and clones are Neisseria specific T-cell lines and clones generated according to the method of the first aspect of the invention, as set out above.

The proteins may be fractionated by SDS-PAGE. The fractions are preferably tested for their ability to stimulate the individual T-cell lines and clones. Preferably fractions containing T-cell stimulants are further characterised by SDS-PAGE.

Polyclonal antibodies may be raised to the T-cell stimulating fraction proteins. The antibodies are preferably used to screen a genomic meningococcal or gonococcal expression library. Preferably the expression library is a  $\lambda$ ZapII library. Isolated neisserial polypeptides which react with the antibodies and their respective DNA fragments are preferably further characterised and sequenced.



In a third aspect, the present invention provides a method of detecting CD4<sup>+</sup> T-cell stimulating recombinant proteins, the method comprising screening a genomic meningococcal or gonococcal expression library for recombinant proteins which stimulate T-cell lines and clones.

Preferably the T-cell lines and clones are meningococcal and/or gonococcal specific T-cell lines and clones generated according to the method of the first aspect of the invention, as set out above.

Preferably the genomic meningococcal or gonococcal expression library is a  $\lambda$ ZapII phage library expressing genomic DNA extracted from a strain of *Neisseria meningitidis* or a strain of *Neisseria gonorrhoea*. Preferably a representative pool of recombinant pBluescript SKII plasmid are excised from the phage library and transformed into *E.coli* strain XL1-Blue. Preferably the plasmids are excised into XL1-Blue using a helper phage.

The transformed *E.coli* are preferably cultured in a medium which may contain ampicillin. Meningococcal or gonococcal protein expression is preferably induced by isopropyl-b-D-thio-galactoside.

Preferably the bacteria are heat-killed and sonicated before adding to antigen presenting cells. The expressed proteins are preferably tested for their ability to stimulate the individual T-cell lines and clones. Preferably CD4<sup>+</sup> T-cell stimulating cultures are identified and subcultured. The subcultures are preferably rescreened for T-cell stimulation.

Preferably the CD4<sup>+</sup> T-cell stimulants are identified by sequencing and may be further characterised.

Alternatively the genomic meningococcal or gonococcal expression library is a  $\lambda$ ZapII phage library expressing genomic DNA extracted from a meningococcal or gonococcal genomic lambda phage display library.

In a fourth aspect the present invention provides a method of detecting CD4<sup>+</sup> T-cell stimulating peptides, the method comprising screening meningococcal or gonococcal genomic phage display libraries (PDLs) to identify peptides which stimulate T-cell lines and clones.

Preferably the T-cell lines and clones are meningococcal and/or gonococcal specific T-cell lines and clones generated according to the method of the first aspect of the invention, as set out above.

Preferably the genomic phage display library (PDL) is generated by fragmenting bacterial DNA, cloning and packaging into bacteriophage vectors. Preferably two vectors are used. The first vector preferably displays peptides up to 1200 amino acids which are expressed at low copy numbers. The second vector preferably displays up to 415 copies of a peptide up to 50 amino acids in size.

Preferably the PDLs are amplified in respective *E.coli* hosts. The cells are preferably heat killed before testing for the ability of the peptides to stimulate the T-cell lines and clones.

Preferably CD4<sup>+</sup> T-cell stimulating cultures are identified and subcultured. The subcultures are preferably rescreened for T-cell stimulation.

Preferably the CD4<sup>+</sup> T-cell stimulants are identified by sequencing and may be further characterised.

In a fifth aspect the present invention provides a method of detecting CD4<sup>+</sup> T-cell stimulating recombinant proteins, using a meningococcal or gonococcal genomic lambda phage display library in accordance with the third aspect of the invention, as set out above.

The meningococcal or gonococcal genomic lambda phage display library is preferably constructed by cloning randomly amplified PCR products using

two random primers, each tagged at 5' end to restriction sites, inserting same into a pre-digested vector, and plating by infecting *E.coli*.

Preferably the vector is a lambda phage and is preferably  $\lambda$ pRH825 vector. The amplified and digested DNA fragments are preferably packaged into the lambda phage using a lambda phage packaging kit. Preferably the restriction sites are *SpeI* or *NotI*.

Preferably the DNA inserts in the plaques formed are sequenced, thereby confirming that the plaques contain DNA fragments of meningococcal or gonococcal origin.

In a sixth aspect the present invention provides the use of a polypeptide in the manufacture of a vaccine against neisserial disease, the peptide comprising an amino acid sequence as shown in SEQIDNO1 and SEQIDNO2 or an active derivative thereof.

Preferably the polypeptide is a CD4<sup>+</sup> T-cell stimulant.

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In a seventh aspect the present invention provides the use of a polypeptide in the manufacture of a vaccine against neisserial disease, the peptide comprising an amino acid sequence as shown in SEQIDNO3 and SEQIDNO4 or an active derivative thereof.

Preferably the polypeptide is a CD4<sup>+</sup> T-cell stimulant.

In an eighth aspect the invention provides a composition for use as a vaccine against neisserial disease, the composition comprising two peptides with the amino acid sequences as shown in SEQIDNO1 and SEQIDNO2, and SEQIDNO3 and SEQIDNO4 or active derivatives thereof.

In a ninth aspect of the present invention there is provided a nucleotide

sequence comprising a base sequence as shown in SEQIDNO1, or an active derivative thereof, the sequence coding for a polypeptide having an amino acid sequence as shown in SEQIDNO1 and SEQIDNO2, or an active derivative thereof.

In a tenth aspect of the present invention there is provided a nucleotide sequence comprising a base sequence as shown in SEQIDNO3, or an active derivative thereof, the sequence coding for a polypeptide having an amino acid sequence as shown in SEQIDNO3 and SEQIDNO4, or an active derivative thereof.

Embodiments of the invention will now be described by way of example only and with reference to the accompanying drawings and sequences, in which:-

Fig. 1 is a graph illustrating the proliferation responses of peripheral blood lymphocytes (PBLs) of three patients and a healthy donor to meningococcal proteins.

Fig. 2 is a graph illustrating the proliferation indices of a T-cell line with fraction (SI-V) of meningococcal proteins separated by SDS PAGE.

Fig. 3 is a graph illustrating the proliferation indices of a T-cell line to subfractions A, B, C and D of section SI in Fig. 2, and also the proliferation index of concanavalin A (Con A) and whole cell lysate of iron-depleted meningococci (SD-).

SEQIDNO1 shows the nucleotide base sequence and the corresponding amino acid sequence of a gene and a polypeptide encoded thereby, according to one aspect of the present invention;

SEQIDNO2 shows the polypeptide sequence of SEQIDNO1;

SEQIDNO3 shows the nucleotide base sequence and the corresponding amino acid sequence of a gene and a polypeptide encoded thereby, according to another aspect of the present invention; and

SEQIDNO4 shows the polypeptide sequence of SEQIDNO3.

In order to identify meningococcal CD4<sup>+</sup> T-cell-stimulating peptides we adopted a number of different programmes all of which involve screening meningococcal peptide antigens, using meningococcal-specific CD4<sup>+</sup> T-cell lines and clones. These lines and clones have been generated over the past three years or so, from the peripheral blood of normal donors and patients recovering from invasive meningococcal disease. In Nottingham, we are in a unique and ideal position to study *in-vivo* primed human T-cells obtained from naturally infected patients, with fresh peripheral blood samples obtained from patients at different stages of illness, namely the acute stage (on admission), early convalescence (seven days after admission), late convalescence (six weeks after discharge) and after full recovery (3 months and twelve months after discharge). We have already generated T-cell lines and clones, specific to meningococcal proteins from the peripheral blood of eight patients recovering from meningococcal disease (P1-8) and eleven healthy donors (D1-11). The healthy donors were identified among twenty five volunteers by testing their peripheral blood lymphocyte (PBL) proliferation in response to meningococcal proteins.

#### **Lymphocyte proliferation assays:**

Briefly, PBLs were isolated from heparinised blood samples by centrifugation over Histopaque (Sigma). The PBLs were washed and cultured in 96-well tissue culture plates at  $2 \times 10^5$  cells/well in RPMI medium containing 10% human AB serum (RPMI-AB). Meningococcal proteins (from strain SD, B:15:P1,16) were prepared by growing the organism under iron restriction, to induce the expression of iron-regulated proteins which are also expressed *in vivo* [Ala'Aldeen, 1994]. The meningococcal proteins (SD-), antigens from *Candida albicans* (a recall antigen) or phytohaemagglutinin (PHA, positive control) were added to quadruplicate wells. RPMI-AB alone (with no antigen) was added to quadruplicate wells to serve as the background. After five days all cultures were pulsed with 1  $\mu$ Ci of tritiated thymidine and incorporation of thymidine was determined after another eighteen hours. A positive response was defined as a PBL proliferation index of at least 2 (see Fig. 1).

Continuous T-cell lines were established by culturing PBLs with the meningococcal proteins and Interleukin 2 (IL-2) for five days, and activated T-cell blasts were stimulated to proliferate by a further nine days culture with IL-2 only. The lines were then maintained by weekly stimulation with proteins in the presence of feeder cells and IL-2. Autologous Epstein-Barr virus transformed B-lymphocytes (EBVB) were used as antigen-presenting feeder cells following irradiation (6000R).

T-cell clones are defined here as the population of cells which originate from a single T-cell. Single T-cell receptors (TCRs) can engage with an extraordinary small number of peptide-HLA complexes ( $<10/\text{cell}$ ) [Valitute, 1995], therefore T-cell clones will provide a highly sensitive system by which it will be possible to detect the presence of peptide antigens within mixtures of proteins. T-cell lines, specific to meningococcal antigens, were seeded at 0.3 cell/well in 96-well tissue culture plates in the presence of irradiated (non-proliferating) autologous EBVB feeder cells, plus low doses of IL-2 [Sinigaglia, 1991]. Cell growth was detected microscopically after one-two weeks and growing cells expanded further by stimulation with meningococcal proteins. All T-cell lines and clones were assessed for the phenotype (and ascertained to be CD4<sup>+</sup> T-cells), using flow cytometry and CD4, CD8 and  $\alpha/\beta$ - and  $\gamma/\delta$ - TCR-specific monoclonal antibodies. Their specificity to meningococcal proteins were tested by measurement of tritiated thymidine incorporation in response to stimulation with meningococcal proteins compared to irrelevant antigens e.g. tetanus toxoid. Large numbers T-cell lines, oligoclones and clones from patients and normal donors have been identified and stored in liquid nitrogen until further use.

### **T-cell responses to fractionated meningococcal proteins**

Meningococcal proteins were fractionated according to their molecular weights by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Two methods were used to prepare the separated proteins for addition to the T-cell cultures:

a) Fractionated proteins were transferred onto nitrocellulose membranes which were transversely divided into five equal sections labelled SI-V, containing proteins of approximate molecular weight range >130 kDa, 70-130 kDa, 50-70 kDa, 34-50 kDa and <34 kDa, respectively. Membranes were then solubilised with dimethyl sulphoxide and tested for their ability to stimulate T-cells using the established meningococcal specific T-cell lines. Using one of the cell lines, section SI (which contained proteins >130 kDa) caused greater T-cell proliferation than any of the other sections (Fig. 2). T-cell lines fed with either EBV-B-cells or fresh autologous PBLs consistently gave similar results.

b) In the second method, SDS-gels containing the fractionated proteins were cut into transverse sections corresponding to the five fractions obtained by the nitrocellulose membrane method. The proteins were then directly eluted from the gel sections and purified by precipitation with organic solvents. This enabled us to measure protein concentrations in each fraction and confirm that whether differences in protein concentration were not responsible for the differences observed in Figure 2. Equivalent concentrations of purified proteins were used in lymphocyte proliferation assays. The results were consistent with those of the nitrocellulose membrane blot method (not shown).

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Section SI consists of more than 12 proteins as seen on silver stained gels, ranging from 130-599 kDa (not shown). Therefore, it was subdivided into four fractions, FIA-D, and their proteins were eluted from gels as described above. The eluted proteins were tested for their ability to stimulate T-cell proliferation. As shown in Figure 3, using T-cell line of a patient, fractions FIC and D induced extremely high T-cell proliferation indices ( $\leq 30$ ), higher than fractions FIA and FIB, the whole of SI or the total SD-protein preparation. Another T-cell line showed the highest T-cell stimulation indices with fraction FIB and FIC, followed by FID, possibly reflecting the HLA specific response.

FIC was chosen for further characterisation and silver staining of SDS-gels showed that it contains four distinct protein bands (not shown). Rabbit polyclonal antibodies were raised to eluted FIC proteins and used to screen an

already established genomic expression ( $\lambda$  Zap II) library. Several reactive meningococcal polypeptides and their respective DNA fragments were isolated. Two of the most promising ones (TspA and TspB) were further studied. The DNA fragments were sequenced and with help from the Sanger-released genomic sequences which were produced by the Neisseria Meningitidis Sequencing Group at the Sanger Centre and can be obtained from <ftp://ftp.sanger.ac.uk/pub/AAREADME.release-policy.txt>, the genes encoding these two proteins were then constructed (see SEQIDNO1-4) and cloned into high expression vectors.

The exact sequences can vary among different isolates of meningococci due to the nature of the organism and its ability to mutate any gene any time. This is a universal problem inherent with any gene of these *Neisseria* organisms. Equivalent genes with homologous sequences exist in *Neisseria gonorrhoea*, as detected on the recently released gonococcal genomic sequence data obtained on the Internet from Oklahoma University, U.S.A.

Western blot experiments on TspA and TspB, using human convalescent sera, confirmed that both proteins are expressed in-vivo and stimulate B-cells following natural infection. The cloned proteins also induced strong CD4<sup>+</sup> T-cell stimulatory effect in our T-cell proliferation assays. These indicate very clearly that they are promising vaccine candidates.

Finally, fractions FIB and FID, and Section SII and SV which produced net-positive T-cell stimulatory effects may consists of many T-cell stimulatory antigens (Fig. 1 and 3), and will be examined.

#### **Detection of T-cell antigens by phage-expression cloning**

Over the past two years or so, we have also established a robust screening system for the identification of CD4<sup>+</sup> T-cell stimulating recombinant proteins, using an expression cloning protocol, which involves screening genomic meningococcal expression libraries.



## 1. $\lambda$ ZapII Expression Library

This method had been successfully applied in other organisms to identify helper T-cell epitopes [Sanderson, 1995; Mougneau, 1995]. Briefly, we used an existing  $\lambda$ ZapII phage library expressing genomic DNA extracted from strain SD N. *meningitidis* [Palmer, 1993 #214]. The library contains  $2 \times 10^5$  recombinants with an average size of insert of 2.3 kb (range up to 10 kb). A representative pool of recombinant pBluescript SKII plasmid were excised (*in vivo*) from the phage library and transformed into *E. coli* strain XL1-Blue, using ExAssist helper phage (Stratgene) as described previously [Ala'Aldeen, 1996; Palmer, 1993].

Transformed *E. coli* with the pBluescript plasmid carrying meningococcal genes were diluted in selective culture media (containing ampicillin) and put in 96-well microtitre plates at 20-30 transformants/wells. The plates were incubated overnight at 37°C with shaking and replicate cultures were made by splitting the overnight cultures, and the original master plates stored at 4°C. The splits were grown in epindorfs for 2-3 hours in fresh medium to  $OD_{600}=0.3$ , then incubated for an additional 2h with 1mM isopropyl-b-D-thio-galactoside (IPTG) to induce meningococcal protein expression. Bacteria were heat-killed, sonicated and added to the antigen presenting cells, and tested for their ability to stimulate individual T-cell lines and clones. Negative controls were sonicates of the same *E. coli* strain transformed with pBluescript SKII with no meningococcal DNA insert. Strong T-cell stimulating wells were identified and their corresponding reference wells diluted and subcultured. Up to 100 single colonies (representing single organisms with single plasmids) were isolated and re-screened for T-cell stimulation. Only potent T-cell stimulants were saved and further pursued. This approach proved highly rewarding, and so far we have identified and further characterised two, previously unknown, potent T-cell stimulating meningococcal polypeptides.

## 2. T-cell antigen detection using phage display libraries (PDL)

Displaying foreign peptides on the surface of bacteriophages is a relatively new but well-established technology. This is different from the normal phage libraries which carry the cloned genes and express and release the proteins inside a host bacterium and not on their own outer coat. In phage display libraries, displayed peptides are encoded as DNA inserts in the structural gene for one of the viral coat proteins and will then appear on the surface of the phage capsid. There are several phage display systems available, each with specific advantages. For example, some are filamentous and others are lytic, some are used as random display libraries (non-specific) which may be used to detect mimotopes, and others are more specific genomic libraries. It is important to note that most phage display libraries have been probed with antibodies in search of specific peptides. Here, we adopted a highly novel approach involving the use of T-cell lines/clones to screen two different meningococcal genomic PDLs to identify good T-cell stimulating peptides. As far as we are aware, these have not been done before.

a) T7Select1 and T7Select415 PDL

One of the novel lytic bacteriophages is Novagen's T7Select Phage Display System which is easy to use and has the capacity to display peptides up to 1200 amino acids, equivalent to 3.6 kb, with protein molecular weight over 100kDa. Such high molecular weight proteins are usually expressed at low copy numbers by T7Select1. Phage T7Select415, however, is capable of displaying up to 415 copies of a peptide up to 50 amino acids in size. Phage assembly occurs in the *E. coli* cytoplasm and mature phages are released by cell lysis. The latter process occurs within a few hours of infection, which makes the system very rapid. To create a genomic display library, meningococcal DNA will be fragmented to appropriate sizes and cloned and packaged into both T7Select1 and T7Select415 vectors as described in the Novagen's T7Select System manual [Novagen, 1996]. This dual approach should allow us to screen for both large and small polypeptides.

A representative population of these PDLs expressing meningococcal

proteins will be diluted and distributed as oligoclonal into 96-well microtitre plates. To each well, appropriate *E. coli* host strains (BL21 for T7Select415 and BLT5403 for T7Select1) will be added to amplify the diluted phage population in these wells. The plates will be split into identical duplicates, one of which will be stored as the reference, and the other heat-killed and tested for the ability to stimulate the T-cell lines/clones as described above for the  $\lambda$ ZAPII library.

b)  $\lambda$ prH825 random meningococcal epitope display library

Another system to utilise is the display of proteins and small peptides on a modified lambda capsid protein D. This protein, which is of 11 kDa with 405 copies expressed as trimers on the phage head [Sternberg, 1995; Mikawa, 1996], is capable of an efficient display of foreign peptides that are fused to its amino- or carboxy-termini [Mikawa, 1996]. This system was successfully used to display a Hepatitis C genomic cDNA library [Alter, 1995] and, more recently, to generate a randomly amplified genomic PDL of known organisms [Lambert, 1993; Kwong-Kowk, 1996; Tomei, 1993]. This involves generating randomly amplified DNA fragments of a known DNA template, using short (random) oligonucleotide primers in polymerase chain reaction (PCR). In collaboration with the Istituto di Ricerche di Biologia Molecolare (IRBM) in Rome, Italy, we have recently constructed a meningococcal genomic lambda phage display library by cloning randomly amplified PCR products into  $\lambda$ prH825 vector, using two random primers, each tagged at 5' end to SpeI or NotI restriction sites to facilitate insertion into the predigested vector. Packaging amplified and digested DNA fragments into lambda phage was performed using a lambda packaging kit (Pharmacia Biotech) and plated by infection of the *E. coli* strain BB4. This yielded  $5 \times 10^7$  plaques, of which a sample of 100 pfu were randomly chosen, and their DNA inserts sequenced. Sequence alignment of the obtained sequence data with those available for *N. Meningitidis* (Sanger, Wellcome) and/or *N. Gonorrhoea*, confirmed that all the chosen plaques contained DNA fragments of meningococcal origin. The fragment sizes ranged from 100-200 bp, representing deduced peptides of up to 60 amino acids long. This PDL was

prepared and established in IRBM and is now ready to be used for the identification of CD4<sup>+</sup> T-cell stimulating recombinant peptides, using the same cloning technique described for the  $\lambda$ ZapII phage system.

We have adopted several selection criteria to focus the search on relevant, potent and promiscuous T-cell epitopes.

Initially, only candidate peptides, which are likely to contain multiple T-cell epitopes that are immunogenic for CD4<sup>+</sup> Th-cells (not CD8<sup>+</sup> T-cells) and presented on MHC class II (HLA-DR, DQ or DP in humans) are studied. Only T-helper (Th) antigens, that bind to a number of widely ranging HLA-types, will be selected. It will be determined whether each patient's CD4<sup>+</sup> Th-response to a candidate meningococcal peptide is due to an established memory Th population (CD45RO<sup>+</sup>) or to activation of naive T-cells (CD45RA<sup>+</sup>). Peptide candidates which activate either the Th2 subset of CD4<sup>+</sup> T-cell or the Th1 subset will be selected. The therapeutic efficacy of both Th1 and Th2-inducing candidate peptides will be evaluated. T-cell clones specific for candidate antigens will be amplified and used to identify the individual T-cell epitopes.

In order to identify and then characterise core epitopes of each candidate peptide, progressively smaller fragments of the DNA will be cloned, expressed and further examined for T-cell stimulation. To define epitopes more accurately, short overlapping peptides representing the defined T-cell stimulating subunits will be synthesised and re-examined. Then N- and C-terminal truncated analogs of the most immunogenic peptide fragment will be synthesised and tested likewise. Finally, alanine scanning mutational analysis will be employed to identify critical amino acid positions responsible for both TCR contact and HLA-class II contact. Here, a series of peptide analogs of the core epitope identified after N- and C-terminal truncation are synthesised, each with a single alanine substituted at successive amino acid positions, and effects on T-cell immunogenicity and on HLA-binding are assessed [Nelson, 1996]. The isotype of class II HLA molecule restriction specificity will be identified for each T-cell clone by antibody blocking experiments.

As a part of the characterisation of the identified proteins, we will study the diversity of these proteins among various strains of meningococci. We have a large collection of clinical isolates of meningococci, the proteins of these strains will be purified (from the gels or clones), tested for T-cell stimulatory capacity and characterised in a way similar to that used for strain SD. Proteins that are expressed in all or more of these stains will be focused on.

#### Identification of HLA restriction

To determine whether different HLA class II molecules present different parts of individual proteins, one of two methods will be used. The protein sub-fragments and their overlapping peptides described above will be tested for their capacity to stimulate T-cell clones generated from different individuals (volunteers or patients). Alternatively, lymphocyte donors will be HLA typed, and the association of responsiveness to particular proteins (or epitopes) and certain alleles of HLA-DR, -DQ or -DP will be determined.

A central aim is to identify T-cell immunogens of *N. meningitidis* which will stimulate T-cell help for the production of protective anti-meningococcal antibodies. Having identified dominant T-cell antigens amongst the proteins, their ability to stimulate T-cell help for antibody production will be investigated *in vivo* in animals and in an *in vitro* immunisation system which has been established and optimised in our laboratories [Davenport, 1992]. Protein fragments or peptides that stimulate T-cells from individuals covering a range of HLA types will be studied for the presence of B-cell epitopes. If the protein contains B-cell epitopes then antibodies from individuals naturally immune to meningococcal disease should recognise these proteins in immunoblots or ELISA. If no B-cell epitopes are recognised then the identified T-cell epitopes will be conjugated to previously characterised B-cell immunogens such as the meningococcal capsular polysaccharides, the class (1, 2/3) proteins, the transferrin binding proteins ... etc.

Whilst endeavouring in the foregoing specification to draw attention to

the features of the invention believed to be of particular importance it should be understood that the Applicant claims protection in respect of any patentable feature hereinbefore referred to and/or shown in the drawings whether or not particular emphasis has been placed thereon.

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## SEQUENCE LISTING

(1) Information for SEQIDNO1:

(a) Sequence Characteristics:

- (i) Length : 2761 base pairs
- (ii) Type : Nucleic acid
- (iii) Strandedness : Double

(b) Molecule type : DNA (genomic)

(c) Original Source:

- (i) Organism : *Neisseria meningitidis*
  - (ii) Strain : SD, serogroup B (B:15:Pl.16)
-

(2) Information for SEQIDNO2:

(a) Sequence Characteristics:

- (i) Length : 880 amino acids
- (ii) Type : amino acid
- (iii) Topology : linear

(b) Molecule type : protein

(c) Original Source:

- (i) Organism : *Neisseria meningitidis*
- (ii) Strain : SD, serogroup B (B:15:Pl.16)



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(3) Information for SEQIDNO3:

(a) Sequence Characteristics:

- (i) Length : 1639 base pairs
- (ii) Type : Nucleic acid
- (iii) Strandedness : Double

(b) Molecule type : DNA (genomic)

(c) Original Source:

- (i) Organism : *Neisseria meningitidis*
  - (ii) Strain : SD, serogroup B (B:15:Pl.16)
-

(4) Information for SEQIDNO4:

(a) Sequence Characteristics:

- (i) Length : 504 amino acids
- (ii) Type : amino acid
- (iii) Topology : linear

(b) Molecule type : protein

(c) Original Source:

- (i) Organism : *Neisseria meningitidis*
- (ii) Strain : SD, serogroup B (B:15:Pl.16)

CAGATGTTTT	TGAAAAAAGA	TGGAGATATG	GATTGATG	CAAATATGCA	18
GAATGATTGC	CGTTTAAAT	GTGGCGGCGG	TCATAATTTG	TAAAAACGGC	68
			TCGGTACATT	CACATAT'TAA	118
ATG CCC GCC GGC CGA CTG CCC CGC CGA TGC CCG ATG ATG ACG AAA	163				
Met Pro Ala Gly Arg Leu Pro Arg Arg Cys Pro Met Met Thr Lys					
1	-5		-10		-15
TTT ACA GAC TGT ACG CGG TCA AAC CGT ATT CAG CCG CCA ACC CAC	208				
Phe Thr Asp Cys Thr Arg Ser Asn Arg Ile Gln Pro Pro Thr His					
	-20		-25		-30
AGG GGA TAC ATC TTG AAA AAC AAC AGA CAA ATC AAA CTG ATT GCC	253				
Arg Gly Tyr Ile Leu Lys Asn Asn Arg Gln Ile Lys Leu Ile Ala					
	-35		-40		-45
GCC TCC GTC GCA GTT GCC GCA TCC TTT CAG GCA CAT GCT GGA CTG	298				
Ala Ser Val Ala Val Ala Ala Ser Phe Gln Ala His Ala Gly Leu					
	-50		-55		-60
GGC GGA CTG AAT ATC CAG TCC AAC CTT GAC GAA CCC TTT TCC GGC	343				
Gly Gly Leu Asn Ile Gln Ser Asn Leu Asp Glu Pro Phe Ser Gly					
	-65		-70		-75
AGC ATT ACC GTA ACC GGC GAA GAA GCC AAA GCC CTG CTA GGC GGC	388				
Ser Ile Thr Val Thr Gly Glu Glu Ala Lys Ala Leu Leu Gly Gly					
	-80		-85		-90
GGC AGC GTT ACC GTT TCC GAA AAA GGC CTG ACC GCC AAA GTC CAC	433				
Gly Ser Val Thr Val Ser Glu Lys Gly Leu Thr Ala Lys Val His					
	-95		100		105
AAG TTG GGC GAC AAA GCC GTC ATT GCC GTT TCT TCC GAA CAG GCA	478				
Lys Leu Gly Asp Lys Ala Val Ile Ala Val Ser Ser Glu Gln Ala					
	110		115		120
GTC CGC GAT CCC GTC CTG GTA TTC CGC ATC GGC GCA GGC GCA CAG	524				
Val Arg Asp Pro Val Leu Val Phe Arg Ile Gly Ala Gly Ala Gln					
	125		130		135
GTA CGG GAA TAC ACC GCC ATC CTC GAT CCT GTC GGC TAC TCG CCC	568				
Val Arg Glu Tyr Thr Ala Ile Leu Asp Pro Val Gly Tyr Ser Pro					
	140		145		150
AAA ACC AAA TCT GCA CTT TCA GAC GGC AAG ACA CAC CGC AAA ACC	613				
Lys Thr Lys Ser Ala Leu Ser Asp Gly Lys Thr His Arg Lys Thr					
	155		160		165
GCT CCG ACA GCA GAG TCC CAA GAA AAT CAA AAC GCC AAA GCC CTC	658				
Ala Pro Thr Ala Glu Ser Gln Glu Asn Gln Asn Ala Lys Ala Leu					
	170		175		180
CGC AAA ACC GAT AAA AAA GAC AGC GCG AAC GCA GCC GTC AAA CCG	703				
Arg Lys Thr Asp Lys Lys Asp Ser Ala Asn Ala Ala Val Lys Pro					
	185		190		195
GCG TAC AAC GGC AAA ACC CAT ACC GTC CGC AAA GGC GAA ACG GTC	748				
Ala Tyr Asn Gly Lys Thr His Thr Val Arg Lys Gly Glu Thr Val					
	200		205		210
AAA CAG ATT GCC GCC GCC ATC CGC CCG AAA CAC CTG ACG CTC GAA	793				
Lys Gln Ile Ala Ala Ala Ile Arg Pro Lys His Leu Thr Leu Glu					
	215		220		225
CAG GTT GCC GAT GCG CTG CTG AAG GCA AAC CCA AAT GTT TCC GCA	838				
Gln Val Ala Asp Ala Leu Leu Lys Ala Asn Pro Asn Val Ser Ala					
	230		235		240
CAC GGC AGA CTG CGT GCG GGC AGC GTG CTT CAC ATT CCG AAT CTG	883				

His Gly Arg Leu Arg	245	Gly Ser Val Leu	250	His Ile Pro	255	Leu	255
AAC AGG ATC AAA GCG GAA CAA CCC AAA CCG CAA ACG GCG AAA CCC	928	Asn Arg Ile Lys Ala Glu Gln Pro Lys Pro Gln Thr Ala Lys Pro	260	265	270		
AAA GCC GAA ACC GCA TCC ATG CCG TCC GAA CCG TCC AAA CAG GCA	973	Lys Ala Glu Thr Ala Ser Met Pro Ser Glu Pro Ser Lys Gln Ala	275	280	285		
ACG GTA GAG AAA CCG GTT GAA AAA CCT GAA GCA AAA GTT GCC GCG	1018	Thr Val Glu Lys Pro Val Glu Lys Pro Glu Ala Lys Val Ala Ala	290	295	300		
CCC GAA GCA AAA GCG GAA AAA CCG GCC GTT CGA CCC GAA CCT GTA	1063	Pro Glu Ala Lys Ala Glu Lys Pro Ala Val Arg Pro Glu Pro Val	305	310	315		
CCC GCT GCA AAT ACT GCC GCA TCG GAA ACC GCT GCC GAA TCC GCC	1108	Pro Ala Ala Asn Thr Ala Ala Ser Glu Thr Ala Ala Glu Ser Ala	320	325	330		
CCC CAA GAA GCC GCC GCT TCT GCC ATC GAC ACG CCG ACC GAC GAA	1153	Pro Gln Glu Ala Ala Ala Ser Ala Ile Asp Thr Pro Thr Asp Glu	335	340	345		
ACC GGT AAC GCC GTT TCC GAA CCT GTC GAA CAG GTT TCT GCC GAA	1198	Thr Gly Asn Ala Val Ser Glu Pro Val Glu Gln Val Ser Ala Glu	350	355	360		
GAA GAA ACC GAA AGC GGA CTG TTC GGC GGT TCG TAC ACC TTG CTG	1243	Glu Glu Thr Glu Ser Gly Leu Phe Gly Gly Ser Tyr Thr Leu Leu	365	370	375		
CTT GCC GGC GGA GGC GCG GCA TTG ATC GCC CTG CTG CTG CTT TTG	1288	Leu Ala Gly Gly Gly Ala Ala Leu Ile Ala Leu Leu Leu Leu	380	385	390		
CGC CTT GCC CAA TCC AAA CGC GCG CGC CGT ACC GAA GAA TCC GTC	1333	Arg Leu Ala Gln Ser Lys Arg Ala Arg Arg Thr Glu Glu Ser Val	395	400	405		
CCT GAG GAA GAG CCT GAC CTT GAC GAC GCG GCA GAC GAC GGC ATA	1378	Pro Glu Glu Glu Pro Asp Leu Asp Asp Ala Ala Asp Asp Gly Ile	410	415	420		
GAA ATC ACC TTT GCC GAA GTC GAA ACT CCG GCA ACG CCC GAA CCC	1423	Glu Ile Thr Phe Ala Glu Val Glu Thr Pro Ala Thr Pro Glu Pro	425	430	435		
GCT CCG AAA AAC GAT GTA AAC GAC ACA CTT GCC TTA GAT GGG GAA	1468	Ala Pro Lys Asn Asp Val Asn Asp Thr Leu Ala Leu Asp Gly Glu	440	445	450		
TCT GAA GAA GAG TTG TCG GCA AAA CAA ACG TTC GAT GTC GAA ACC	1513	Ser Glu Glu Glu Leu Ser Ala Lys Gln Thr Phe Asp Val Glu Thr	455	460	465		
GAT ACG CCT TCC AAC CGC ATC GAC TTG GAT TTC GAC AGC CTG GCA	1558	Asp Thr Pro Ser Asn Arg Ile Asp Leu Asp Phe Asp Ser Leu Ala	470	475	480		
GCC GCG CAA AAC GGC ATT TTA TCC GGC GCA CTT ACG CAG GAT GAA	1603	Ala Ala Gln Asn Gly Ile Leu Ser Gly Ala Leu Thr Gln Asp Glu	485	490	495		
GAA ACC CAA AAA CGC GCG GAT GCC GAT TGG AAC GCC ATC GAA TCC	1648	Glu Thr Gln Lys Arg Ala Asp Ala Asp Trp Asn Ala Ile Glu Ser	500	505	510		

ACA GAC AGC GTG TAC GAG GAG ACC TTC AAC CCG TAC AAC CT 1693

Thr Asp Ser Val Tyr Glu Pro Glu Thr Phe Asn Pro Tyr Asn Pro  
515 520 525

GTC GAA ATC GTC ATC GAC ACG CCC GAA CCG GAA TCT GTC GCC CAA 1738  
Val Glu Ile Val Ile Asp Thr Pro Glu Pro Glu Ser Val Ala Gln  
530 535 540

ACT GCC GAA AAC AAA CCG GAA ACC GTC GAT ACC GAT TTC TCC GAC 1783  
Thr Ala Glu Asn Lys Pro Glu Thr Val Asp Thr Asp Phe Ser Asp  
545 550 555

AAC CTG CCC TCA AAC AAC CAT ATC GGC ACA GAA GAA ACA GCT TCC 1828  
Asn Leu Pro Ser Asn Asn His Ile Gly Thr Glu Glu Thr Ala Ser  
560 565 570

GCA AAA CCT GCC TCA CCC TCC GGA CTG GCA GGC TTC CTG AAG GCT 1873  
Ala Lys Pro Ala Ser Pro Ser Gly Leu Ala Gly Phe Leu Lys Ala  
575 580 585

TCC TCG CCC GAA ACC ATC TTG GAA AAA ACA GTT GCC GAA GTC CAA 1918  
Ser Ser Pro Glu Thr Ile Leu Glu Lys Thr Val Ala Glu Val Gln  
590 595 600

ACA CCG GAA GAG TTG CAC GAT TTC CTG AAA GTG TAC GAA ACC GAT 1963  
Thr Pro Glu Glu Leu His Asp Phe Leu Lys Val Tyr Glu Thr Asp  
605 610 615

GCC GTC GCG GAA ACT GCG CCT GAA ACG CCC GAT TTC AAC GCC GCC 2008  
Ala Val Ala Glu Thr Ala Pro Glu Thr Pro Asp Phe Asn Ala Ala  
620 625 630

GCA GAC GAT TTG TCC GCA TTG CTT CAA CCT GCC GAA GCA CCG TCC 2053  
Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ala Glu Ala Pro Ser  
635 640 645

GTT GAG GAA AAT ATA ACG GAA ACC GTT GCC GAA ACA CCC GAC TTC 2098  
Val Glu Glu Asn Ile Thr Glu Thr Val Ala Glu Thr Pro Asp Phe  
650 655 660

AAC GCC ACC GCA GAC GAT TTG TCC GCA TTA CTT CAA CCT TCT GAA 2143  
Asn Ala Thr Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ser Glu  
665 670 675

GTA CCT GCC GTT GAG GAA AAT GCA GCG GAA ATC GTT GCC GAT GAT 2188  
Val Pro Ala Val Glu Glu Asn Ala Ala Glu Ile Val Ala Asp Asp  
680 685 690

TTG TCC GCA CTG TTG CAA CCT GCT GAA GCA CCG GCT GTT GAG GAA 2233  
Leu Ser Ala Leu Leu Gln Pro Ala Glu Ala Pro Ala Val Glu Glu  
695 700 705

AAT GTA ACG GAA ACT GTT GCC GAA ACG TCC GAC TTC CAC ACC GCC 2278  
Asn Val Thr Glu Thr Val Ala Glu Thr Ser Asp Phe His Thr Ala  
710 715 720

GCA GAC GAT TTG TCC GCA CTG TTG CAA CCT GCT GAA GTA CCG GCC 2323  
Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ala Glu Val Pro Ala  
725 730 735

GTT GAG GAA AAT GTA ACG AAA ACC GTT GCC GAA ATA CCT GAT TTC 2368  
Val Glu Glu Asn Val Thr Lys Thr Val Ala Glu Ile Pro Asp Phe  
740 745 750

AAC GCC ACC GCA GAC GAT TTG TCC GCA TTA CTT CAA CCT TCT GAA 2413  
Asn Ala Thr Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ser Glu  
755 760 765

GTA CCG GCC GTT GAG GAA AAT GCA GCG GAA ATC ACT TTG GAA ACG 2458  
Val Pro Ala Val Glu Glu Asn Ala Ala Glu Ile Thr Leu Glu Thr  
770 775 780

CCT	GAT	TCC	AAC	ACC	TCC	GAG	GCA	GAC	GCT	TTG	CCC	GAC	TTG	TTG	2503
Pro	Asp	Ser	Asn	Thr	Ser	Glu	Ala	Asp	Ala	Leu	Pro	Asp	Phe	Leu	
				785					790					795	
AAA	GAC	GGC	GAG	GAG	GAA	ACG	GTA	GAT	TGG	AGC	ATC	TAC	CTC	TCG	2548
Lys	Asp	Gly	Glu	Glu	Glu	Thr	Val	Asp	Trp	Ser	Ile	Tyr	Leu	Ser	
				800					805					810	
GAA	GAA	AAT	ATC	CCA	AAT	AAT	GCA	GAT	ACC	AGT	TTC	CCT	TCG	GAA	2593
Glu	Glu	Asn	Ile	Pro	Asn	Asn	Ala	Asp	Thr	Ser	Phe	Pro	Ser	Glu	
				815					820					825	
TCT	GTA	GGT	TCT	GAC	GCG	CCT	TCC	GAA	GCG	AAA	TAC	GAC	CTT	GCC	2638
Ser	Val	Gly	Ser	Asp	Ala	Pro	Ser	Glu	Ala	Lys	Tyr	Asp	Leu	Ala	
				830					835					840	
GAA	ATG	TAT	CTC	GAA	ATC	GGC	GAC	CGC	GAT	GCC	GCT	GCC	GAG	ACA	2683
Glu	Met	Tyr	Leu	Glu	Ile	Gly	Asp	Arg	Asp	Ala	Ala	Ala	Glu	Thr	
				845					850					855	
GTG	CAG	AAA	TTG	CTG	GAA	GAA	GCG	GAA	GGC	GAC	GTA	CTC	AAA	CGT	2728
Val	Gln	Lys	Leu	Leu	Glu	Glu	Ala	Glu	Gly	Asp	Val	Leu	Lys	Arg	
				860					865					870	
GCC	CAA	GCA	TTG	GCG	CAG	GAA	TTG	GGT	ATT	TGA					2761
Ala	Gln	Ala	Leu	Ala	Gln	Glu	Leu	Gly	Ile	Stop					
				875					880						

Met	Pro	Ala	Gly	Arg	Leu	Pro	Arg	Arg	Cys	Pro	Met	Met	Thr	Lys	1	-5	-10	-15
Phe	Thr	Asp	Cys	Thr	Arg	Ser	Asn	Arg	Ile	Gln	Pro	Pro	Thr	His	-20	-25	-30	
Arg	Gly	Tyr	Ile	Leu	Lys	Asn	Asn	Arg	Gln	Ile	Lys	Leu	Ile	Ala	-35	-40	-45	
Ala	Ser	Val	Ala	Val	Ala	Ala	Ser	Thr	Gln	Ala	His	Ala	Gly	Leu	-50	-55	-60	
Gly	Gly	Leu	Asn	Ile	Gln	Ser	Asn	Leu	Asp	Glu	Pro	Phe	Ser	Gly	-65	-70	-75	
Ser	Ile	Thr	Val	Thr	Gly	Glu	Glu	Ala	Lys	Ala	Leu	Leu	Gly	Gly	-80	-85	-90	
Gly	Ser	Val	Thr	Val	Ser	Glu	Lys	Gly	Leu	Thr	Ala	Lys	Val	His	-95	100	105	
Lys	Leu	Gly	Asp	Lys	Ala	Val	Ile	Ala	Val	Ser	Ser	Glu	Gln	Ala	110	115	120	
Val	Arg	Asp	Pro	Val	Leu	Val	Phe	Arg	Ile	Gly	Ala	Gly	Ala	Gln	125	130	135	
Val	Arg	Glu	Tyr	Thr	Ala	Ile	Leu	Asp	Pro	Val	Gly	Tyr	Ser	Pro	140	145	150	
Lys	Thr	Lys	Ser	Ala	Leu	Ser	Asp	Gly	Lys	Thr	His	Arg	Lys	Thr	155	160	165	
Ala	Pro	Thr	Ala	Glu	Ser	Gln	Glu	Asn	Gln	Asn	Ala	Lys	Ala	Leu	170	175	180	
Arg	Lys	Thr	Asp	Lys	Lys	Asp	Ser	Ala	Asn	Ala	Ala	Val	Lys	Pro	185	190	195	
Ala	Tyr	Asn	Gly	Lys	Thr	His	Thr	Val	Arg	Lys	Gly	Glu	Thr	Val	200	205	210	
Lys	Gln	Ile	Ala	Ala	Ala	Ile	Arg	Pro	Lys	His	Leu	Thr	Leu	Glu	215	220	225	
Gln	Val	Ala	Asp	Ala	Leu	Leu	Lys	Ala	Asn	Pro	Asn	Val	Ser	Ala	230	235	240	
His	Gly	Arg	Leu	Arg	Ala	Gly	Ser	Val	Leu	His	Ile	Pro	Asn	Leu	245	250	255	
Asn	Arg	Ile	Lys	Ala	Glu	Gln	Pro	Lys	Pro	Gln	Thr	Ala	Lys	Pro	260	265	270	
Lys	Ala	Glu	Thr	Ala	Ser	Met	Pro	Ser	Glu	Pro	Ser	Lys	Gln	Ala	275	280	285	
Thr	Val	Glu	Lys	Pro	Val	Glu	Lys	Pro	Glu	Ala	Lys	Val	Ala	Ala	290	295	300	
Pro	Glu	Ala	Lys	Ala	Glu	Lys	Pro	Ala	Val	Arg	Pro	Glu	Pro	Val	305	310	315	
Pro	Ala	Ala	Asn	Thr	Ala	Ala	Ser	Glu	Thr	Ala	Ala	Glu	Ser	Ala	320	325	330	
Pro	Gln	Glu	Ala	Ala	Ala	Ser	Ala	Ile	Asp	Thr	Pro	Thr	Asp	Glu	335	340	345	

Thr Gly Asn Ala Val	Ser	Glu Pro Val	Glu Gln Val Ser	Ala	Glu
350			355		360
Glu Glu Thr Glu Ser	Gly Leu Phe Gly	Gly Ser Tyr Thr	Leu Leu		
365		370	375		
Leu Ala Gly Gly	Gly Ala Ala Leu Ile	Ala Leu Leu Leu Leu	Leu		
380		385	390		
Arg Leu Ala Gln Ser	Lys Arg Ala Arg	Arg Thr Glu Glu Ser	Val		
395		400	405		
Pro Glu Glu Glu Pro	Asp Leu Asp Asp	Ala Ala Asp Asp Gly	Ile		
410		415	420		
Glu Ile Thr Phe Ala	Glu Val Glu Thr	Pro Ala Thr Pro Glu	Pro		
425		430	435		
Ala Pro Lys Asn Asp	Val Asn Asp Thr	Leu Ala Leu Asp Gly	Glu		
440		445	450		
Ser Glu Glu Glu Leu	Ser Ala Lys Gln	Thr Phe Asp Val Glu	Thr		
455		460	465		
Asp Thr Pro Ser Asn	Arg Ile Asp Leu	Asp Phe Asp Ser Leu	Ala		
470		475	480		
Ala Ala Gln Asn Gly	Ile Leu Ser Gly	Ala Leu Thr Gln Asp	Glu		
485		490	495		
Glu Thr Gln Lys Arg	Ala Asp Ala Asp	Trp Asn Ala Ile Glu	Ser		
500		505	510		
Thr Asp Ser Val Tyr	Glu Pro Glu Thr	Phe Asn Pro Tyr Asn	Pro		
515		520	525		
Val Glu Ile Val Ile	Asp Thr Pro Glu	Pro Glu Ser Val Ala	Gln		
530		535	540		
Thr Ala Glu Asn Lys	Pro Glu Thr Val	Asp Thr Asp Phe Ser	Asp		
545		550	555		
Asn Leu Pro Ser Asn	Asn His Ile Gly	Thr Glu Glu Thr Ala	Ser		
560		565	570		
Ala Lys Pro Ala Ser	Pro Ser Gly Leu	Ala Gly Phe Leu Lys	Ala		
575		580	585		
Ser Ser Pro Glu Thr	Ile Leu Glu Lys	Thr Val Ala Glu Val	Gln		
590		595	600		
Thr Pro Glu Glu Leu	His Asp Phe Leu	Lys Val Tyr Glu Thr	Asp		
605		610	615		
Ala Val Ala Glu Thr	Ala Pro Glu Thr	Pro Asp Phe Asn Ala	Ala		
620		625	630		
Ala Asp Asp Leu Ser	Ala Leu Leu Gln	Pro Ala Glu Ala Pro	Ser		
635		640	645		
Val Glu Glu Asn Ile	Thr Glu Thr Val	Ala Glu Thr Pro Asp	Phe		
650		655	660		
Asn Ala Thr Ala Asp	Asp Leu Ser Ala	Leu Leu Gln Pro Ser	Glu		
665		670	675		
Val Pro Ala Val Glu	Glu Asn Ala Ala	Glu Ile Val Ala Asp	Asp		
680		685	690		
Leu Ser Ala Leu Leu	Gln Pro Ala Glu	Ala Pro Ala Val Glu	Glu		



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sn Val Thr Glu Thr Val Ala Glu Thr Ser Asp Phe His Thr Ala	710	715	720
Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ala Glu Val Pro Ala	725	730	735
Val Glu Glu Asn Val Thr Lys Thr Val Ala Glu Ile Pro Asp Phe	740	745	750
Asn Ala Thr Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ser Glu	755	760	765
Val Pro Ala Val Glu Glu Asn Ala Ala Glu Ile Thr Leu Glu Thr	770	775	780
Pro Asp Ser Asn Thr Ser Glu Ala Asp Ala Leu Pro Asp Phe Leu	785	790	795
Lys Asp Gly Glu Glu Glu Thr Val Asp Trp Ser Ile Tyr Leu Ser	800	805	810
Glu Glu Asn Ile Pro Asn Asn Ala Asp Thr Ser Phe Pro Ser Glu	815	820	825
Ser Val Gly Ser Asp Ala Pro Ser Glu Ala Lys Tyr Asp Leu Ala	830	835	840
Glu Met Tyr Leu Glu Ile Gly Asp Arg Asp Ala Ala Ala Glu Thr	845	850	855
Val Gln Lys Leu Leu Glu Glu Ala Glu Gly Asp Val Leu Lys Arg	860	865	870
Ala Gln Ala Leu Ala Gln Glu Leu Gly Ile Stop	875	880	

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AAATCCGGAG	CTTATCTCGG	AAAT	GTTCGAATTA	CGAACAAGTT	24
TTACGGCTTC	AGGCTGCTGG	GTCTATGGTT	TTAATTCTGT	TTGTCGTTAT	74
		TTAATTCTTT	AAAAGACATA	GGCAAAGTAG	124
TTT TGC AGA ACC AGC AAG AAT GAT GAT CGA ATA ATA AAA TTT AGG					169
Phe Cys Arg Thr Ser Lys Asn Asp Asp Arg Ile Ile Lys Phe Arg					
1	-5		-10	-15	
CCA TCT AAA TTA AAG TTT TTT GAA TCT ACA GGA TAT AGA AAA ATC					214
Pro Ser Lys Leu Lys Phe Phe Glu Ser Thr Gly Tyr Arg Lys Ile					
	-20		-25	-30	
AAT AAT GAA TTT TCT AAA TTC ACA GAA GCG GCA AAT GTC GAA CAT					259
Asn Asn Glu Phe Ser Lys Phe Thr Glu Ala Ala Asn Val Glu His					
	-35		-40	-45	
ATC CCC ACG GGC GCA AAA GCC CGA ATC AAC GCA AAG ATA ACC GCC					304
Ile Pro Thr Gly Ala Lys Ala Arg Ile Asn Ala Lys Ile Thr Ala					
	-50		-55	-60	
AGC GTA TCC CGC GCC GCC GTC TTG TCA GGA GTC GGC AAA CTT GCC					349
Ser Val Ser Arg Ala Ala Val Leu Ser Gly Val Gly Lys Leu Ala					
	-65		-70	-75	
CGC TTA GGC GCG AAA TTA AGC ACA AGG GCA GTT CCT TAT GTC GGA					394
Arg Leu Gly Ala Lys Leu Ser Thr Arg Ala Val Pro Tyr Val Gly					
	-80		-85	-90	
ACA GCC CTT TTA GCC CAT GAC GTA TAC GAA ACT TTC AAA GAA GAC					439
Thr Ala Leu Leu Ala His Asp Val Tyr Glu Thr Phe Lys Glu Asp					
	-95		100	105	
ATA CAG GCA CAA GGC TAC CAA TAC GAC CCC GAA ACC GAC AAA TTT					484
Ile Gln Ala Gln Gly Tyr Gln Tyr Asp Pro Glu Thr Asp Lys Phe					
	110		115	120	
GTA AAA GGC TAC GAA TAT AGT AAT TGC CTT TGG TAC GAA GAC AAA					529
Val Lys Gly Tyr Glu Tyr Ser Asn Cys Leu Trp Tyr Glu Asp Lys					
	125		130	135	
AGA CGT ATT AAT AGA ACC TAT GGC TGC TAC GGC GTT GAC AGT TCG					574
Arg Arg Ile Asn Arg Thr Tyr Gly Cys Tyr Gly Val Asp Ser Ser					
	140		145	150	
ATT ATG CGC CTT ATG TCC GAT GAC AGC AGA TTC CCC GAA GTC AAA					619
Ile Met Arg Leu Met Ser Asp Asp Ser Arg Phe Pro Glu Val Lys					
	155		160	165	
GAA TTG ATG GAA AGC CAA ATG TAT AGG CTG GCA CGT CCG TTT TGG					664
Glu Leu Met Glu Ser Gln Met Tyr Arg Leu Ala Arg Pro Phe Trp					
	170		175	180	
AAT TGG CAT AAA GAA GAA CTG AAT AAA TTA AGT TCT TTG GAT TGG					709
Asn Trp His Lys Glu Glu Leu Asn Lys Leu Ser Ser Leu Asp Trp					
	185		190	195	
AAT AAT TTT GTT TTA AAT CGT TGC ACA TTT AAT TGG AAT GGC GGA					754
Asn Asn Phe Val Leu Asn Arg Cys Thr Phe Asn Trp Asn Gly Gly					
	200		205	210	
GAT TGT TTG GTC AAT AAA GGT GAT GAT TTC AGA AAT GGG GCT GAT					799
Asp Cys Leu Val Asn Lys Gly Asp Asp Phe Arg Asn Gly Ala Asp					
	215		220	225	
TTT TCC CTT ATT CGC AAT TCA AAA TAC AAA GAA GAA ATG GAT GCC					844
Phe Ser Leu Ile Arg Asn Ser Lys Tyr Lys Glu Glu Met Asp Ala					
	230		235	240	

AAA AAG CTG GAA GAG ATA TCG TTG AAA GTC GAT GCC AAT TCC 889  
 Lys Lys Leu Glu Glu Ile Leu Ser Leu Lys Val Asp Ala Asn Pro  
 245 250 255

GAC AAA TAC ATA AAG GAA ACC GGT TAT CCC GGT TAT TCC GAA AAA 934  
 Asp Lys Tyr Ile Lys Glu Thr Gly Tyr Pro Gly Tyr Ser Glu Lys  
 260 265 270

GTA GAA GTC GCA CCC GGA ACA AAA GTG AAT ATG GGT CCC GTC ACG 979  
 Val Glu Val Ala Pro Gly Thr Lys Val Asn Met Gly Pro Val Thr  
 275 280 285

GAC AGG AAC GGG AAT CCC GTT CAG GTT GTC GCA ACA TTC GGC AGG 1024  
 Asp Arg Asn Gly Asn Pro Val Gln Val Val Ala Thr Phe Gly Arg  
 290 295 300

GAT TCG CAA GGC AAC ACC ACG GTG GAT GTT CAA GTA ATC CCG CGT 1069  
 Asp Ser Gln Gly Asn Thr Thr Val Asp Val Gln Val Ile Pro Arg  
 305 310 315

CCC GAC TTG ACC CCC GGA AGC GCG GAA GCA CCG AAC GCA CAG CCG 1114  
 Pro Asp Leu Thr Pro Gly Ser Ala Glu Ala Pro Asn Ala Gln Pro  
 320 325 330

CTG CCC GAA GTA TCG CCC GCC GAA AAC CCC GCA AAC AAC CCG AAC 1159  
 Leu Pro Glu Val Ser Pro Ala Glu Asn Pro Ala Asn Asn Pro Asn  
 335 340 345

CCC AAT GAG AAC CCC GGC ACG AGC CCC AAT CCC GAA CCC GAC CCC 1204  
 Pro Asn Glu Asn Pro Gly Thr Ser Pro Asn Pro Glu Pro Asp Pro  
 350 355 360

GAT TTG AAT CCC GAT GCA AAT CCC GAT ACG GAC GGA CAG CCC GGC 1249  
 Asp Leu Asn Pro Asp Ala Asn Pro Asp Thr Asp Gly Gln Pro Gly  
 365 370 375

ACA AGA CCC GAT TCC CCC GCC GTT CCG GGA CGC ACA AAC GGC AGG 1294  
 Thr Arg Pro Asp Ser Pro Ala Val Pro Gly Arg Thr Asn Gly Arg  
 380 385 390

GAC GGC AAA GAC GGA AAG GAC GGC AAA GAT GGC GGC CTT TTG TGC 1339  
 Asp Gly Lys Asp Gly Lys Asp Gly Lys Asp Gly Gly Leu Leu Cys  
 395 400 405

AAA TTC TTC CCC GAC ATT CTC GCT TGC GAC AGG CTG CCC GAG TCC 1384  
 Lys Phe Phe Pro Asp Ile Leu Ala Cys Asp Arg Leu Pro Glu Ser  
 410 415 420

AAT CCG GCA GAA GAT TTA AAT CTG CCG TCT GAA ACC GTC AAT GTA 1429  
 Asn Pro Ala Glu Asp Leu Asn Leu Pro Ser Glu Thr Val Asn Val  
 425 430 435

GAG TTT CAG AAA TCA GGA ATC TTT CAA GAT TCC GCA CAG TGT CCC 1474  
 Glu Phe Gln Lys Ser Gly Ile Phe Gln Asp Ser Ala Gln Cys Pro  
 440 445 450

GCA CCT GTC ACT TTC ACA GTG ACT GTG CTT GAT TCA AGC AGG CAG 1519  
 Ala Pro Val Thr Phe Thr Val Thr Val Leu Asp Ser Ser Arg Gln  
 455 460 465

TTC GCG TTC AGC TTT GAG AAC GCA TGT ACC ATA GCC GAA CGG CTA 1564  
 Phe Ala Phe Ser Phe Glu Asn Ala Cys Thr Ile Ala Glu Arg Leu  
 470 475 480

AGG TAC ATG CTT CTC GCC CTT GCT TGG GCG GTT GCC GCC TTT TTT 1609  
 Arg Tyr Met Leu Leu Ala Leu Ala Trp Ala Val Ala Ala Phe Phe  
 485 490 495

TGT ATC CGC ACA GTA TCT CGT GAA GTC TAG 1639  
 Cys Ile Arg Thr Val Ser Arg Glu Val Stop  
 500 504

Phe Cys Arg Thr Ser Lys Asn Asp Asp Arg Ile Ile Lys Phe Arg	1	-5	-10	-15
Pro Ser Lys Leu Lys Phe Phe Glu Ser Thr Gly Tyr Arg Lys Ile	-20	-25	-30	
Asn Asn Glu Phe Ser Lys Phe Thr Glu Ala Ala Asn Val Glu His	-35	-40	-45	
Ile Pro Thr Gly Ala Lys Ala Arg Ile Asn Ala Lys Ile Thr Ala	-50	-55	-60	
Ser Val Ser Arg Ala Ala Val Leu Ser Gly Val Gly Lys Leu Ala	-65	-70	-75	
Arg Leu Gly Ala Lys Leu Ser Thr Arg Ala Val Pro Tyr Val Gly	-80	-85	-90	
Thr Ala Leu Leu Ala His Asp Val Tyr Glu Thr Phe Lys Glu Asp	-95	100	105	
Ile Gln Ala Gln Gly Tyr Gln Tyr Asp Pro Glu Thr Asp Lys Phe	110	115	120	
Val Lys Gly Tyr Glu Tyr Ser Asn Cys Leu Trp Tyr Glu Asp Lys	125	130	135	
Arg Arg Ile Asn Arg Thr Tyr Gly Cys Tyr Gly Val Asp Ser Ser	140	145	150	
Ile Met Arg Leu Met Ser Asp Asp Ser Arg Phe Pro Glu Val Lys	155	160	165	
Glu Leu Met Glu Ser Gln Met Tyr Arg Leu Ala Arg Pro Phe Trp	170	175	180	
Asn Trp His Lys Glu Glu Leu Asn Lys Leu Ser Ser Leu Asp Trp	185	190	195	
Asn Asn Phe Val Leu Asn Arg Cys Thr Phe Asn Trp Asn Gly Gly	200	205	210	
Asp Cys Leu Val Asn Lys Gly Asp Asp Phe Arg Asn Gly Ala Asp	215	220	225	
Phe Ser Leu Ile Arg Asn Ser Lys Tyr Lys Glu Glu Met Asp Ala	230	235	240	
Lys Lys Leu Glu Glu Ile Leu Ser Leu Lys Val Asp Ala Asn Pro	245	250	255	
Asp Lys Tyr Ile Lys Glu Thr Gly Tyr Pro Gly Tyr Ser Glu Lys	260	265	270	
Val Glu Val Ala Pro Gly Thr Lys Val Asn Met Gly Pro Val Thr	275	280	295	
Asp Arg Asn Gly Asn Pro Val Gln Val Val Ala Thr Phe Gly Arg	290	295	300	
Asp Ser Gln Gly Asn Thr Thr Val Asp Val Gln Val Ile Pro Arg	305	310	315	
Pro Asp Leu Thr Pro Gly Ser Ala Glu Ala Pro Asn Ala Gln Pro	320	325	330	
Leu Pro Glu Val Ser Pro Ala Glu Asn Pro Ala Asn Asn Pro Asn	335	340	345	

Pro	Asn	Glu	Asn	Pro	Gly	Thr	Ser	Pro	Asn	Pro	Glu	Pro	Asp	Pro	350	355	360
Asp	Leu	Asn	Pro	Asp	Ala	Asn	Pro	Asp	Thr	Asp	Gly	Gln	Pro	Gly	365	370	375
Thr	Arg	Pro	Asp	Ser	Pro	Ala	Val	Pro	Gly	Arg	Thr	Asn	Gly	Arg	380	385	390
Asp	Gly	Lys	Asp	Gly	Lys	Asp	Gly	Lys	Asp	Gly	Gly	Leu	Leu	Cys	395	400	405
Lys	Phe	Phe	Pro	Asp	Ile	Leu	Ala	Cys	Asp	Arg	Leu	Pro	Glu	Ser	410	415	420
Asn	Pro	Ala	Glu	Asp	Leu	Asn	Leu	Pro	Ser	Glu	Thr	Val	Asn	Val	425	430	435
Glu	Phe	Gln	Lys	Ser	Gly	Ile	Phe	Gln	Asp	Ser	Ala	Gln	Cys	Pro	440	445	450
Ala	Pro	Val	Thr	Phe	Thr	Val	Thr	Val	Leu	Asp	Ser	Ser	Arg	Gln	455	460	465
Phe	Ala	Phe	Ser	Phe	Glu	Asn	Ala	Cys	Thr	Ile	Ala	Glu	Arg	Leu	470	475	480
Arg	Tyr	Met	Leu	Leu	Ala	Leu	Ala	Trp	Ala	Val	Ala	Ala	Phe	Phe	485	490	495
Cys	Ile	Arg	Thr	Val	Ser	Arg	Glu	Val	Stop						500	504	



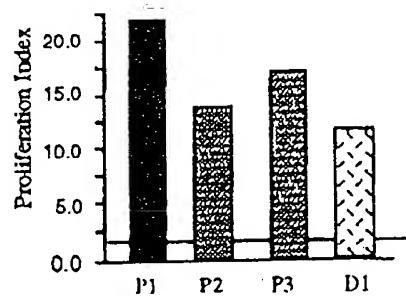


Fig 1

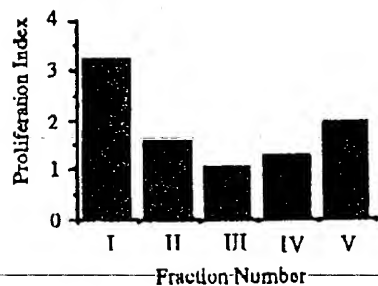


Fig 2

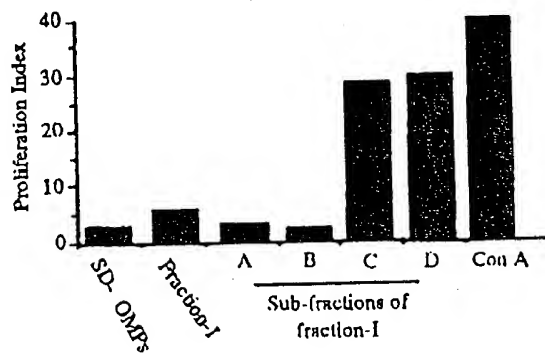


Fig 3

PCT NO : 8899 / 02.205

FORM 23/77 : 9/7/99

AGENT : Swindell & Pearson